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Involvement of the TREM-1 / DAP12 pathway in the innate immune responses to *Porphyromonas gingivalis*

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Abbreviations

Triggering Receptor Expressed on Myeloid cells 1: TREM-1, DNAX activating protein of 12 kDa: DAP12, qPCR: quantitative real-time polymerase chain reaction, Toll-like receptor: TLR, interleukin: IL.

Abstract

Porphyromonas gingivalis, is a Gram-negative obligate oral anaerobic bacterium highly implicated in periodontal disease, the most prevalent chronic inflammatory disease, but recent evidence also indicates a potential contribution to systemic inflammation. The Triggering Receptor Expressed on Myeloid cells 1 (TREM-1) is a cell surface receptor of the immunoglobulin superfamily, which, along with its adaptor signalling molecule DAP12, is involved in immune response to bacterial and fungal infections, particularly by amplifying the production of pro-inflammatory cytokines by the host. The aim of the present study was to investigate the effect of *P. gingivalis* on the expression of the TREM-1 / DAP12 pathway, as well as its engagement in pro-inflammatory cytokine production, by the myelomonocytic cell line MonoMac-6. *P. gingivalis* enhanced TREM-1 gene expression by the cells, concomitantly to an increase of soluble TREM-1 secretion. Engagement of TREM-1, by introducing anti-TREM-1 to the experimental system, resulted in further potentiation of the pro-inflammatory responses to *P. gingivalis*, as evaluated by a further enhancement of interleukin (IL)-1β and IL-6 secretion. On the contrary, the synthetic TREM-1 antagonist LP17 reduced the *P. gingivalis*-induced IL-1β and IL-6 secretion by approximately 50%. In conclusion, the putative periodontal pathogen *P. gingivalis* can positively regulate the expression of the TREM-1 / DAP12 pathway in monocytic cells. Moreover, engagement of TREM-1 can further potentiate the pro-inflammatory responses to *P. gingivalis* infection. This effect may contribute not only to the pathogenesis of inflammatory periodontal disease, but also to the enhancement of systemic inflammation.

Keywords: TREM-1, DAP12, LP17, MonoMac-6, *Porphyromonas gingivalis*, periodontal disease, cytokines, inflammation, innate immunity.

1. Introduction

Porphyromonas gingivalis, is a Gram-negative obligate anaerobic bacterium, which is associated with periodontitis, the most prevalent chronic inflammatory disease in humans (Darveau, 2011). *P. gingivalis* induces locally a chronic inflammatory response of the tooth-supporting (periodontal tissues), that eventually results in alveolar bone destruction, which is a hallmark of the disease. If left untreated, periodontitis may culminate in tooth loss. Elevated levels of *P. gingivalis* are detected in periodontal lesions, and these can be significantly reduced following successful periodontal therapy (Cappelli et al., 2009; Salvi et al., 1997; Socransky et al., 2002; Ximenez-Fyvie et al., 2000; Yamazaki et al., 2004). Apart from its well accepted role as an oral pathogen in the establishment of chronic inflammation at the site of infection, there is increasing evidence of a link between *P. gingivalis*-associated periodontal disease and systemic inflammatory conditions, such as diabetes, preterm birth, aspiration pneumonia and atherosclerotic cardiovascular disease (Brodala et al., 2005; Liao et al., 2009; Lin et al., 2003; Maekawa et al., 2011; Pussinen and Mattila, 2004). On the cellular level, a convincing body of evidence indicates that *P. gingivalis* possesses unique signalling properties, which enable it to evade, subvert or manipulate the host immune defences by exploiting the toll-like receptor (TLR) family, in particular TLR2 (Burns et al., 2006; Hajishengallis et al., 2002; Hajishengallis et al., 2008b; Harokopakis et al., 2006; Wang et al., 2010). As an opportunistic pathogen, *P. gingivalis* can preferentially target innate immunity, which may in turn disable adaptive immunity, hence causing a general deregulation of the immune responses (Hajishengallis, 2009; Hajishengallis et al., 2008a). Whether inflammation is beneficial or not for *P. gingivalis* is a matter of controversy. At early stages of infection, an immunosuppressive effect of *P. gingivalis* may be beneficial for allowing its colonization of the host tissues, while evading the surveillance mechanisms of innate immunity. At later stages of infection, induction of inflammation by *P. gingivalis* may facilitate its increased demands in nutrients.

The Triggering Receptor Expressed on Myeloid cells 1 (TREM-1) is a cell surface receptor of the immunoglobulin superfamily, involved in the innate inflammatory response to bacterial and fungal infections (Bleharski et al., 2003; Bouchon et al., 2000). A crucial role of TREM-1 has initially been demonstrated in the development of septic shock (Bouchon et al., 2001; Gibot et al., 2004b). TREM-1 activation and expression occurs synergistically with TLRs (Arts et al., 2011; Klesney-Tait and Colonna, 2007; Klesney-Tait et al., 2006), as the TREM family contains both inhibitory and activating receptors capable of modulating the signaling downstream of TLRs (Allcock et al., 2003; Ornatowska et al., 2007). Moreover, TREM-1 has also been associated with NOD-Like Receptors (NLR), responsible for sensing microbial danger signals and amplifying the inflammatory response (El Mezayen et al., 2007; Netea et al., 2006). On the molecular level, TREM-1 regulates immune cell function, by forming an intracellular complex with signalling adapter DNAX activating protein of 12 kDa (DAP12) (Bouchon et al., 2000; Colonna and Facchetti, 2003; Ford and McVicar, 2009; Tomasello and Vivier, 2005). DAP12 mediates further molecular recruitment and down-stream signalling that would eventually enhance cytokine production and amplify the inflammatory response. Hence, the TREM-1 / DAP12 signalling system appears to have a central role in innate immunity, by regulating the magnitude of the inflammatory response, upon recognition of the bacterial challenge.

Monocytes are a major source of TREM-1 in inflammation (Arts et al., 2011; Cavaillon, 2009; Ferat-Osorio et al., 2009; Wong-Baeza et al., 2006) and their expression has been shown to be regulated in several bacterial and fungal infections, including infections of the respiratory track, gut or amniotic fluid (Aoki et al., 2004; Begum et al., 2004; Buckland et al., 2011; del Fresno et al., 2008; How et al., 2011; Kusanovic et al., 2011; Richeldi et al., 2004; Schmausser et al., 2008). Individual microbial components, such as lipopolysaccharide (LPS) and peptidoglycan, can cause up-regulation of cell surface-localized TREM-1 by monocytes, as well as release in its soluble (s)TREM-1 form (Begum et al., 2004; Gibot et al.,

2004b; Gomez-Pina et al., 2007; Murakami et al., 2007; Ramanathan et al., 2005; Zeng et al., 2007). The sTREM-1 appears to be released during the course of infection, and may well be a particularly useful marker of systemic inflammation, as demonstrated in systemic sepsis, septic arthritis, pneumonia (Collins et al., 2009; Gibot et al., 2005; Gibot et al., 2004a; Gibot et al., 2004c; Knapp et al., 2004).

Even though the role of TREM-1 in monocyte activation and secretion of pro-inflammatory molecules has been established in a number of microbial diseases, little is known about the regulation of its expression during host cell infection by periodontal pathogens. Since the TREM-1 / DAP12 signalling pathway can amplify inflammation, and since it overlaps, in both positive and negative cross-talks with pathways known to be activated by *P. gingivalis* (Dower et al., 2008; Pathirana et al., 2010), there is merit to investigate whether and how this species may regulate the expression of TREM-1 / DAP12. Hence, by using a human myelomonocytic (MonoMac-6) cell line, the present *in vitro* study aims a) to investigate the effect of *P. gingivalis* on the expression of the TREM-1 / DAP12 pathway, and b) to evaluate the potential involvement of TREM-1 in the pro-inflammatory responses to *P. gingivalis*.

2. Materials and methods

2.1 Bacterial strain and growth conditions

Porphyromonas gingivalis strain W50 (OMZ 308) was grown anaerobically on Columbia Blood Agar (CBA) plates for 3 to 4 days at 37°C, followed by anaerobic sub-culturing for 2 to 3 days at 37°C in Brain Heart Infusion (BHI) broth. The multiplicity of infection (MOI) of the inoculum was confirmed by plating serial dilutions of *P. gingivalis* anaerobically on CBA.

2.2 Cell culture and bacterial challenge of the myelomonocytic cell line MonoMac-6

The myelomonocytic cell line MonoMac-6 was obtained from the German Collection of Microorganisms and Cell Cultures (Mascheroder, Braunschweig, Germany). The cells were cultured in RPMI-Glutamax supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 1% sodium pyruvate (all from Invitrogen, Life Technologies, Basel, Switzerland), and 9 µg/ml bovine insulin (Sigma-Aldrich, Buchs, Switzerland). For the experiments, 1 x 10^6 cells/ml were cultured in antibiotics-free medium, in the presence or absence of *P. gingivalis*, at multiplicity of infection (MOI, cell:bacteria ratio) 1, 10 and 100, for 4 h or 24 h. Experiments were carried out in triplicate cultures, and at least three independent experiments were performed. A representative experiment is shown.

2.3 Bacterial viability assay

To investigate the bacterial viability under the cell culture conditions, *P. gingivalis* W50 MOI 100 was re-suspended in antibiotic-free cell-culture medium (RPMI-Glutamax and 10% FBS), in co-culture with MonoMac-6 cells, as indicated in the previous section. Upon completion of the experiments (after 4 h or 24 h), 50 µl of serially diluted cell culture supernatants were plated on CBA and cultured anaerobically. The revival of *P. gingivalis* was evaluated by counting the colony-forming units (CFU)s on the plates, and calibrating the number against ml of culture media. Three independent experiments were performed. Results from one representative experiment are provided as mean CFU ± standard deviation (SD) of triplicate cultures.

2.4 Cytotoxicity assay

Potential cytotoxicity induced in MonoMac-6 cells by *P. gingivalis* infection was determined by measuring the extracellularly released lactate dehydrogenase (LDH) activity using the CytoTox 96 Non-radioactive Cytotoxicity Assay (Promega, Mannheim, Germany). For the experiments, MonoMac-6 cells were challenged with *P. gingivalis* MOI 10 and 100, for 24 h. Upon completion of the experiments, 50 µl of the cell-free culture supernatant were

transferred onto an optically clear 96-well plate, and processed according to the manufacturer's instructions. The absorbance was read at 490 nm by a spectophotometric plate reader (Epoch, BioTek, Luzern, Switzerland). The enzyme activity released from damaged cells into the supernatant was expressed as a percentage of total (intracellular + extracellular) LDH activity. The values provided represent mean percentage ± (SD) from three independent experiments.

2.5 RNA extraction and cDNA synthesis

After completion of the experiments, the culture supernatants were removed and stored at -80 °C, until further use. The cell monolayers were washed twice in PBS before being lysed. Total RNA was extracted from the collected lysate by using the RNeasy Mini Kit (QIAGEN, Basel, Switzerland), according to the manufacturer's instructions. RNA concentration was measured by a NanoDrop 1000 spectrophotometer. One microgram of total RNA was then reverse transcribed into single-stranded cDNA by using M-MLV Reverse Transcriptase, $Oligo(dT)_{15}$ Primers, and PCR Nucleotide Mix according to the manufacturer's protocol (Promega, Mannheim, Germany), at 40 °C for 60 min, and 70 °C for 15 min. The cDNA was then stored at −20 °C, until further use.

2.6 Quantitative real-time polymerase chain reaction (qPCR)

For gene expression analyses, TaqMan qPCR was performed in an ABI Prism 7000 Sequence Detection System and software (Applied Biosystems, Life Technologies, Basel, Switzerland). Beta-2 microglobulin (B2M) was used as endogenous RNA control in the samples (house-keeping gene). For the amplification reactions, the Applied Biosystems TaqMan Gene Expression Master Mix and Gene Expression Assay kits were used (assay IDs TREM-1: Hs00218624-m1, DAP12: Hs00182426-m1, B2M: Hs00984230-m1). The standard PCR conditions were 10 min at 95 °C, followed 40 cycles at 95 °C for 15 sec and 60 ºC for 1 min.

The expression levels of the target transcripts in each sample were calculated by the comparative Ct method ($2^{-\Delta Ct}$ formula) after normalization to the house-keeping gene.

2.7 Measurement of sTREM-1, IL-1β and IL-6 levels by ELISA

The levels of sTREM-1, as well as IL-1β and IL-6 in the cell-free culture supernatants were measured by commercially available specific enzyme-linked immunosorbent assay (ELISA) kits (DuoSet, R&D Systems, Abingdon, UK). The absorbance at 450 nm was measured using a microplate reader (Epoch, BioTek, Luzern, Switzerland), with a wavelength correction set at 570 nm to subtract background. A standard curve was generated using a four-parameter logistic curve fit for each set of samples assayed.

2.8 TREM-1 engagement experiments

For the engagement of TREM-1 in the pro-inflammatory responses, MonoMac-6 cells were cultured in 96-well flat-bottom plates, pre-coated (4 h at 37°C) with either anti-human TREM-1 antibody (10 µg/ml) or an isotype control (10 µg/ml) (R & D Systems, Abingdon, UK, mouse IgG_1, clone #193015). The cells were plated at a density of 2 x 10^5 cells/well, and challenged with *P. gingivalis* MOI 10 and 100, for 24 h. At the end of the experiments, the cell-free culture supernatants were collected, and the concentrations of IL-1β and IL-6 were measured by ELISA, as described above.

2.9 TREM-1 antagonist experiments

In a separate set of experiments, the relative involvement of TREM-1 in IL-1β and IL-6 production was investigated by the use of its antagonist LP17 (Pepscan Presto B.V., Lelystad, The Netherlands). LP17 is a synthetic peptide mimicking a highly conserved domain of soluble TREM-1. The exact mechanism of LP17 action is not clear, but it may impair TREM-1 dimerization, or act as a decoy receptor competing with the natural ligand of TREM-1 (Gibot et al., 2004b). For the experiments, LP17 was added to the cells at concentrations 10 ng/ml, 50 ng/ml and 100 ng/ml, simultaneously to *P. gingivalis* (MOI 100). A control peptide

containing the same amino acids as LP17, but in different sequence order (Pepscan Presto B.V., Lelystad, The Netherlands), was also used as previously (Gibot et al., 2004b). At the end of the 24 h experiments, the cell-free culture supernatants were collected, and the concentrations of IL-1β and IL-6 were measured by ELISA, as described further above.

2.10 Confocal Laser Scanning Microscopy (CLSM) analysis of the cultures

To investigate the presence of TREM-1 on the cell surface, as well as the localization of *P. gingivalis*, CLSM was used. The cells were cultured on poly-d-lysine-coated dishes (MatTek Corp., Ashton, MA, USA), challenged with *P. gingivalis* MOI 100, for 24 h. Upon completion of the experiments, the dishes were washed and stained using anti-TREM-1 Alexa 488 (R&D Systems, Abington, UK) followed by fixation in 4% paraformaldehyde (PFA) for 10 min at room temperature. Thereafter, they were stained by fluorescent *in situ* hybridization (FISH) for the detection of *P .gingivalis*. For this analysis, two oligonucleotide probes purchased from Microsynth (Balgach, Switzerland), were used simultaneously, one specific for *P. gingivalis* (L-Ping1006-2 labelled at the 5'-end with Cy3), and one universal bacterial probe (EUB338 labelled at the 5'-end with Cy5). The sequences of these probes as well as the standard hybridization conditions are provided elsewhere (Guggenheim et al., 2009; Zijnge et al., 2010). For nuclear staining, the samples were further incubated with DAPI (Invitrogen, Life Technologies, Basel, Switzerland), to counter-stain DNA. The visualization was performed as described previously (Klinke et al., 2011). In brief, stained samples were examined using a DM IRB/E inverted microscope (Leica Mikroskopie, Wetzlar, Germany), fitted with a UV laser and an Ar laser (both from Coherent Inc., Santa Clara, CA, USA), a He-Ne laser (Uniphase Vertriebs, Eching/Munich, Germany), and a TCS SP5 computer-operated confocal laser scanning system (Leica Lasertechnik, Heidelberg, Germany). Filters were set to 430-470 nm to detect DAPI, to 500–540 nm for Alexa 488, and to 660–710 nm for Cy5. Confocal images were obtained using a x 63 (numeric aperture 1.30) glycerol immersion objective. Z-series were generated by vertical optical sectioning with the slice thickness set at

1.02 µm. Image acquisition was performed in x 8 line average mode. Scans were recombined and processed using Imaris 7.3.0 software (Bitplane, Zürich, Switzerland), without any qualitative changes to the raw images.

2.11 Statistical analysis

A two-way analysis of variance (ANOVA) was used to analyze the statistical significances of the results, using Bonferroni post hoc test for comparisons between individual groups. The data were considered significant at $P < 0.05$.

3. Results

MonoMac-6 cell cultures were challenged for 24 h with *P. gingivalis* MOIs of 10 and 100. The potential cytotoxic effect of this challenge was investigated by measuring percentage of extracellularly released LDH activity. There were no significant differences between the unchallenged cell cultures (11.8 ± 1.8 %) and the ones challenged with *P. gingivalis* MOI 10 (12.2 ± 4.2 %), or MOI 100 (15.8 ± 4.3 %).

The survival of *P. gingivalis* under the cell culture conditions was confirmed by plating of the cell culture supernatants onto agar plates, after 4 h and 24 h of challenge. After 4 h, when *P. gingivalis* was cultured alone, the revival was 58 ± 9 x 10^6 CFU/ml, whereas in co-culture with MonoMac-6 cells this was 1.2 ± 0.1 x 10^6 CFU/ml, indicating that the presence of the cells reduced the presence of *P. gingivalis* in the culture. Nevertheless, this difference was diminished after 24 h, as *P. gingivalis* alone was revived at 2.6 ± 0.2 x 10^6 CFU/ml, whereas in co-culture with MonoMac-6 cells this was 2.7 ± 0.1 x 10^6 CFU/ml.

Further on, the effect of *P. gingivalis* on the regulation of TREM-1 and DAP12 gene expression in MonoMac-6 cells was investigated by qPCR. It was found that the cells constitutively expressed these genes (Figure 1). Nevertheless, TREM-1 expression was significantly up-regulated with the highest *P. gingivalis* MOI employed (100), at both 4 h and

24 h post-infection (Figure 1A). DAP12 was constitutively expressed at relatively high levels, compared to TREM-1, and up-regulated only at 24 h, with *P. gingivalis* of MOI 1 and 10. However, this expression declined to control levels with the highest (MOI 100) *P. gingivalis* concentration used (Figure 1B).

As sTREM-1 is released from myeloid cells during the course of infection, it was of merit to further investigate the presence of this molecule in the cell culture supernatants of the *P. gingivalis*-infected MonoMac-6 cells. Interestingly, high levels of sTREM-1 were detected at both 4 h and 24 h post-infection with *P. gingivalis* MOI 100 (Figure 2), as detected by ELISA. Compared to the corresponding control, this enhanced secretion was approximately 3-fold at 4 h and 4-fold at 24 h.

The localization of TREM-1 on the cell surface was also investigated by CLSM. In the unchallenged control cultures, there was a strong positive green staining for TREM-1 associated with the surface of MonoMac-6 cells (Figure 3B). However, when the cells were infected with *P. gingivalis*, the localization of TREM-1 on the cell surface was decreased (Figure 3C). As identified by FISH staining, *P. gingivalis* was localized on or inside the cells, in line with its host cell-invading capacity (Figure 3C, arrows).

In further experimentations, the engagement of TREM-1 was investigated, by evaluating the simultaneous effect of anti-TREM-1 antibodies (the natural ligand for TREM-1) on the stimulation of IL-1β and IL-6 secretion by *P. gingivalis*-challenged cells. After 24 h, *P. gingivalis* induced the secretion of both IL-1β and IL-6 by the cells in a concentration-dependent manner (Figure 4). However, the presence of anti-TREM-1 further enhanced this capacity. The presence of anti-TREM-1 was adequate to cause a significant enhancement of IL-1β secretion by the cells, even in the absence of *P. gingivalis*, as compared to the presence of the IgG isotype control. When the cells were challenged with *P. gingivalis* in concomitant presence of anti-TREM-1, there was a further enhancement of IL-1 β secretion, which was 2.7-fold with MOI 10 and 1.6-fold with MOI 100 (Figure 4A). In the case of IL-6, the

presence of anti-TREM-1 further enhanced the secretion of this cytokine by 5.1-fold and 6-fold, with *P. gingivalis* MOI 10 and MOI 100, respectively (Figure 4B).

Conversely, in an additional set of experimentations, the LP17 antagonist of TREM-1 was introduced into the experimental system simultaneously to *P. gingivalis* (MOI 100) for 24 h. It was first confirmed that LP17 neither induced any cytotoxicity, nor did it affect the growth of *P. gingivalis* during the experimental period (data not shown). Nevertheless, the presence of LP17 resulted in significant reduction of IL-1β and IL-6 production by the *P. gingivalis*-challenged cells, by 45% and 53%, respectively (Table 1). Ascending concentrations of LP17 were used (10 ng/ml -100 ng/ml), yet the lowest concentration was as efficient as the highest one. The control peptide did not affect the capacity of *P. gingivalis* to stimulate IL-1β or IL-6 production by the cells.

4. Discussion

The present study demonstrates a role of the TREM-1 / DAP12 system in *P. gingivalis*-infected monocytic cells, indicating an additional pathway by which *P. gingivalis* may manipulate the innate immune responses. The gene expression of TREM-1, an immunoglobulin receptor with a functional role in the amplification of inflammation, is up-regulated by *P. gingivalis* infection. This finding is in line with the up-regulation of TREM-1 gene expression in murine monocytic cells in response to *P. gingivalis*. (Liang et al., 2009). The present study further considers the involvement of DAP-12 (which is the moiety of this pathway), but also provides confirmation of this effect of *P. gingivalis* in human cells. This validation is particularly important in light of a recent gene expression profiling comparison between human and mouse monocyte subsets, which revealed that among the most striking differential expressions was that of TREM-1 (Ingresoll et al., 2010). In agreement with the findings above, up-regulation of TREM-1 gene expression has also been shown in RAW 264.7 cells challenged with either *Escherichia coli* LPS, or *Pseudomonas aeroginosa*, and this

effect was sustainable over 48 h (Zeng et al., 2007). Nevertheless, in another study it was demonstrated that *E. coli* LPS does not affect TREM-1 transcription in peripheral blood monocytes, but causes an increase in cell surface TREM-1 expression, denoting an effect on post-transcriptional modulation (Wong-Baeza et al., 2006). These seemingly conflicting results could be partly explained by recent evidence using microarray technology, suggesting that there is both positive and negative cross-talk between LPS and TREM-1 signalling in human monocytes (Dower et al., 2008). Moreover, *Salmonella abortus* LPS was shown to cause a transient increase of cell-surface TREM-1 at 6 h, succeeded by an increase of sTREM-1 at 24 h (Gomez-Pina et al., 2007). Accordingly, the TREM-1 surface expression in granulocytes is decreased upon stimulation with *E. coli* LPS, marked by a concomitant increase in sTREM-1 (Knapp et al., 2004). The findings of the present study do demonstrate an increase of sTREM-1 by time, in a similar pattern to TREM-1 gene expression. The reduced TREM-1 detection on the surface of the cells when co-cultured with *P. gingivalis*, as confirmed by CLSM, is also corroborated by the increase in sTREM-1. The present findings are also in agreement with a reduction in murine monocyte TREM-1 surface expression by *P. gingivalis*, as demonstrated earlier by flow cytometry (Liang et al., 2009). This may indicate a shift of cell-bound TREM-1 to its soluble form. It is not clear in the present experimental system how TREM-1 is shed from the cells surface, but could be by a mechanism involving indigenous metalloproteinases (Gomez-Pina et al., 2007). Alternatively, this could account for the cysteine-proteinases (gingipains) of *P. gingivalis*, responsible for cleaving a number of cell surface molecules (Curtis et al., 2001; Guo et al., 2010).

The expression of DAP12 was further investigated in this experimental system. DAP12 is the intracellular adaptor molecule for TREM-1 that conveys the further downstream signalling that regulates inflammation (Bouchon et al., 2000), also crucial for monomyelocytic differentiation (Gingras et al., 2002). The data demonstrates that DAP12 is not affected as early as 4 h, despite the up-regulation of its up-stream TREM-1 with *P.*

gingivalis MOI 100. There little evidence in the literature on the regulation of DAP12 expression in response to bacterial challenge, and hence it is rather difficult to evaluate this response in comparison to other experimental systems. Specifically, it is not clear if the up-regulation of TREM-1 in combination with a steady DAP12 expression is adequate to enhance down-stream signalling for the amplification of the inflammatory response. However, in line with the present findings, *Mycobacterium bovis* was shown to up-regulate DAP12 expression in lung macrophages, but not as strongly as TREM-1 (Aoki et al., 2004). Moreover, lack of DAP12 expression does attenuate the TREM-1-mediated inflammatory response, as demonstrated in a DAP12-deficient mice sepsis model (Turnbull et al., 2005). A noteworthy finding of the present work is that after 24 h, only the lower (MOI 1) and mid-concentrations (MOI 10) of *P. gingivalis* used were able significantly up-regulated DAP12 expression. This observation is well in line with the bi-phasic effects of *P. gingivalis* on the innate immune responses. Since the lower and mid-range concentrations of *P. gingivalis* do induce further TREM-1 expression at the late 24 h time-point, the expression of its intracellular DAP12 adaptor may be enhanced in order to prime or sensitize further the cell in the absence of a strong up-stream signalling. This could also denote an adaptation to the infection, potentially for the establishment of the chronicity of inflammation. This is also well in line with the notion that the TREM-1 / DAP12 pathway does not itself stimulate the production of pro-inflammatory mediators but it rather lowers the threshold of the inflammatory response to bacterial infection (Klesney-Tait et al., 2006).

Engagement of TREM-1 in combination with microbial ligands that activate TLRs can synergistically increase the production of pro-inflammatory cytokines, including IL-1 β, IL-6, IL-8, monocyte chemotactic protein-1 and tumor necrosis factor-α in monocytes and macrophages (Bleharski et al., 2003; Bouchon et al., 2000; Schenk et al., 2005; Schenk et al., 2007), hence amplifying the inflammatory response to bacterial challenge (Netea et al., 2006). *P. gingivalis* has a known capacity to induce pro-inflammatory cytokine production by

monocytes (Bostanci et al., 2007a; Bostanci et al., 2007b; Hamedi et al., 2009), but the potential synergism of TREM-1 has never been investigated. Hence, the TREM-1 engagement approach was evaluated in the present experimental system by introducing anti-TREM-1 antibodies, a natural ligand for TREM-1 receptor. It was indeed demonstrated that this engagement of TREM-1 further enhanced the production of IL-1β and IL-6 by the *P. gingivalis*-challenged cells, in line with the previous studies.

In a further approach, the synthetic antagonist of TREM-1 LP17 was introduced to the experimental system. It was demonstrated that LP17 reduced *P. gingivalis*-induced IL-1β and IL-6 secretion by the cells, by approximately 50%. This documents further the involvement of TREM-1 in the propagation or amplification of inflammatory responses to *P. gingivalis*. However, since the production of these cytokines was not totally abolished to control levels, this finding also denotes that other pathways are still in function, allowing for a basal inflammatory response to *P. gingivalis*. The present findings are in line with a previous study employing LP17, which demonstrated that this peptide reduced by 50% - 70% the capacity of *E. coli* LPS to stimulate IL-1β and tumor necrosis factor (TNF)-α by human monocytes (Gibot et al., 2004b).

5. Conclusions

The present study is the first to address the regulation of TREM-1 / DAP12 pathway, an amplifier of inflammation, in relation to oral infection. It demonstrates that the putative oral pathogen *P. gingivalis* can stimulate the expression of the TREM-1 / DAP12 pathway in monocytic cells, associated with an increased release of sTREM-1, which may constitute a marker of systemic inflammation (Gibot and Cravoisy, 2004). Moreover, TREM-1 engagement can further potentiate the pro-inflammatory responses of monocytes to *P. gingivalis* infection, whereas its inhibition can conversely reduce this. Collectively, these results may denote a positive regulation of the TREM-1 / DAP12 pathway in *P. gingivalis*-

induced inflammatory host responses. This may have implications not only in the pathogenesis of inflammatory periodontal disease, but also in the contribution of *P. gingivalis* to the development of systemic inflammatory responses (Lin et al., 2003; Salvi et al., 1997).

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Tables

Table 1.

Effect of LP17 on IL-1β and IL-6 secretion in response to *P. gingivalis*.

	IL-1β	**IL-6**
Control	11.5 ± 0.6 *	8.3 ± 0.5 *
P.g.	50.6 ± 9.5	64.9 ± 0.5
***P.g.* + LP17 (10 ng/ml)**	28.5 ± 2.5 *	40.2 ± 0.9 *
***P.g.* + LP17 (50 ng/ml)**	34.3 ± 2.3 *	40.3 ± 1.3 *
***P.g.* + LP17 (100 ng/ml)**	27.9 ± 2.3 *	30.3 ± 0.8 *
***P.g.* + control peptide (100 ng/ml)**	45.7 ± 9.4	70.0 ± 1.7

MonoMac-6 cell cultures were exposed to *P. gingivalis* MOI 100 for 24 h, in the presence or absence of ascending LP17 concentrations (10 ng/ml, 50 ng/ml, 100 ng/ml), or its control peptide (100 ng/ml). Upon completion of the experiments, the cell-free culture supernatants were collected and the concentrations of IL-1β and IL-6 were measured by ELISA. Numbers represent mean values ± standard deviations (SD) from triplicate cultures of one representative experiment. The asterisk represents statistically significant difference between the *P. gingivalis* (alone)-challenged group and all other groups.

Figure legends

Figure 1.

Regulation of TREM-1 and DAP12 gene expression in MonoMac-6 cells, in response to *P. gingivalis* infection. MonoMac-6 cell cultures were exposed to *P. gingivalis* MOI 1, 10 or 100

for 4 h and 24 h. Upon completion of the experiments, the gene expression levels of TREM-1 (A) and DAP12 (B) were measured by qPCR analysis, normalized against the expression levels of the house-keeping gene. The results are expressed as the $2^{-\Delta CT}$ formula. Bars represent mean values ± standard errors of mean (SEM) from triplicate cell cultures of one representative experiment. The asterisk represents statistically significant difference between the *P. gingivalis*-challenged and control groups.

Figure 2. Secretion of soluble (s)TREM-1 by MonoMac-6 cells, in response to *P. gingivalis*. MonoMac-6 cell cultures were exposed to *P. gingivalis* MOI 1, 10 or 100 for 4 h and 24 h. Upon completion of the experiments, the concentrations of sTREM-1 secreted into the culture supernatants were measured by ELISA. Bars represent mean values ± standard errors of mean (SEM) from triplicate cultures of one representative experiment. The asterisk represents statistically significant difference between the *P. gingivalis*-challenged and control groups.

Figure 3. Representative confocal laser scanning microscopy (CLSM) images of MonoMac-6 cells infected with *P. gingivalis.* MOI 100 for 24 h. TREM-1 on the cell surface was visualized using anti-TREM-1 (green), and the cell nuclei appear blue due to counter-staining of the DNA with DAPI. Bacteria were stained by FISH with Cy3-labelled L-Pging1006-2 and Cy5-labelled universal bacterial probe EUB338. (A) Control cells, only DAPI stained, (B) Control cells, stained by both DAPI and anti-TREM-1, (C) *P. gingivalis*-infected cells, *P. gingivalis* (yellow) associated with the cells is indicated by the arrows. Scales = 15 µm.

Figure 4. Engagement of TREM-1 in cytokine stimulating-responses of *P. gingivalis*. MonoMac-6 cells were cultured in 96-well plates pre-coated with anti-human TREM-1, or matching IgG_1 isotype control, and exposed to *P. gingivalis* MOI 10 or 100 for 24 h. Upon completion of the experiments, the cell-free culture supernatants were collected and the

concentrations of IL-1β and IL-6 were measured by ELISA. Bars represent mean values ± standard errors of mean (SEM) from triplicate cultures of one representative experiment. The asterisk represents statistically significant difference between the anti-TREM-1 and IgG_1 coated groups.

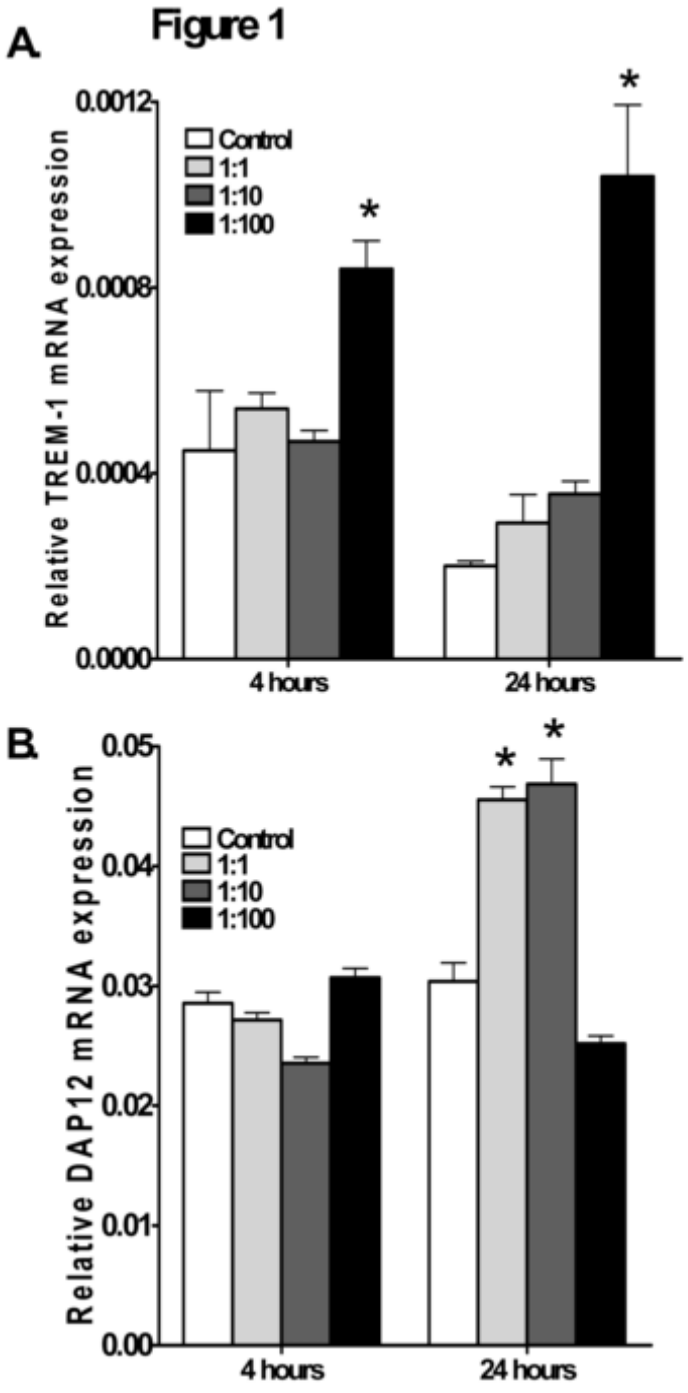


Figure 2

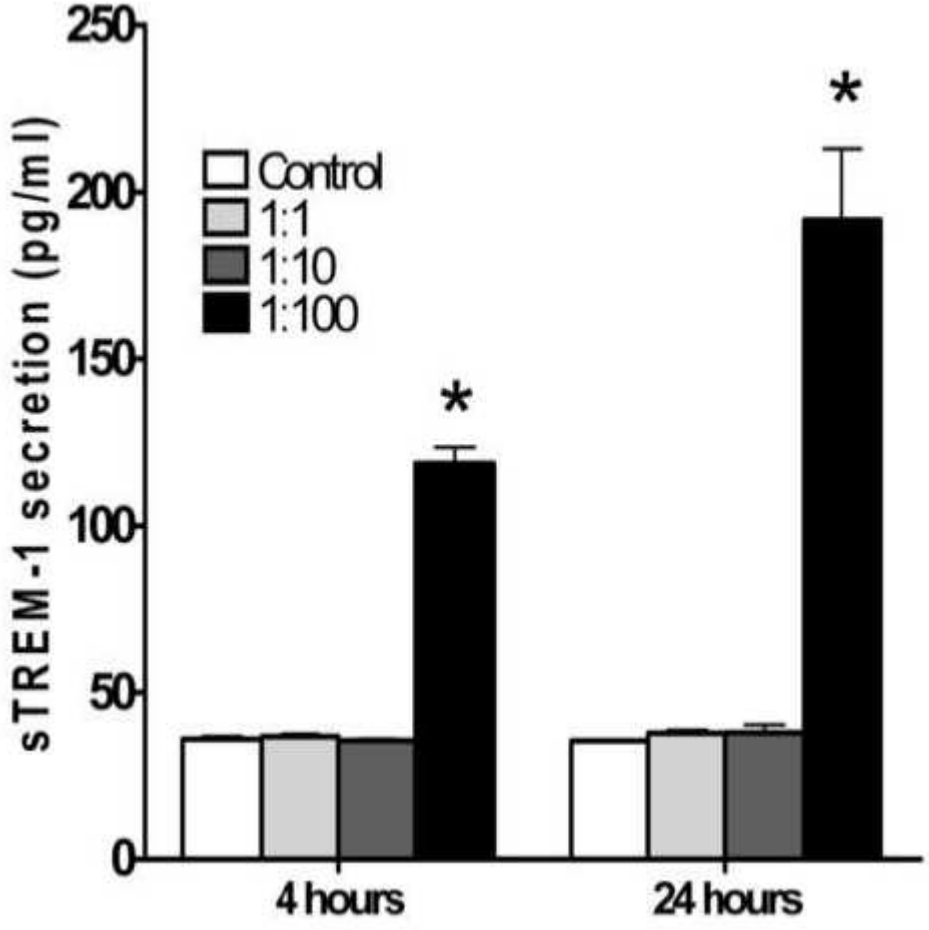

250
200
150
100
50
0
sTREM-1 secretion (pg/ml)
Control
1:1
1:10
1:100
*
*
4 hours
24 hours

Figure 3

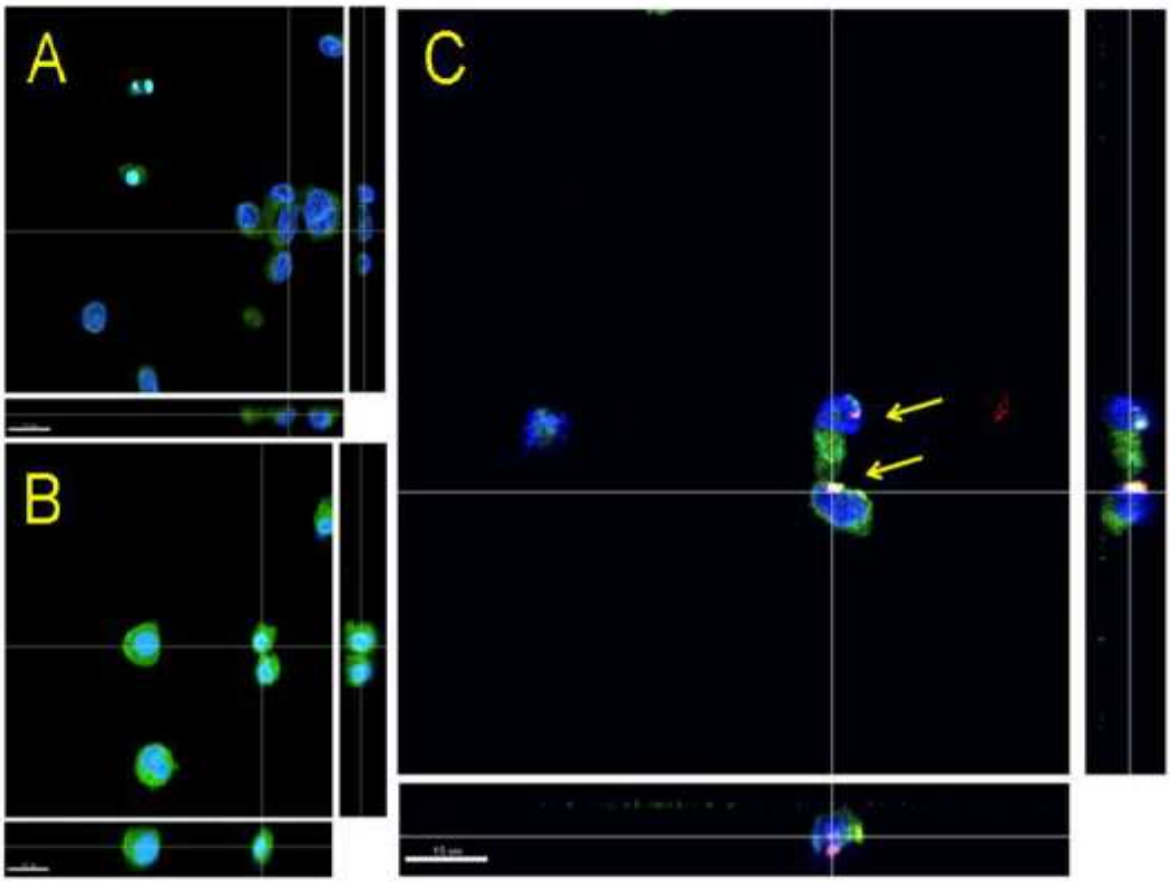
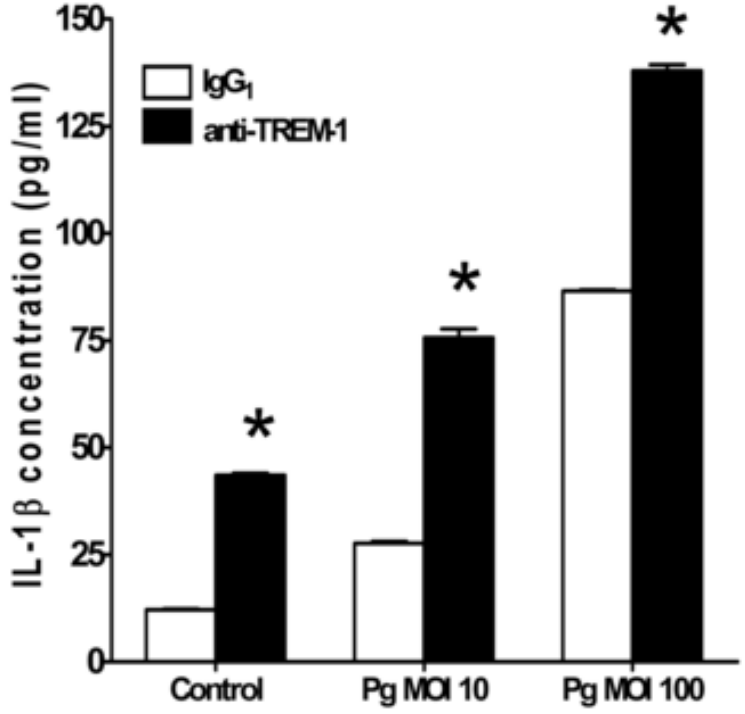
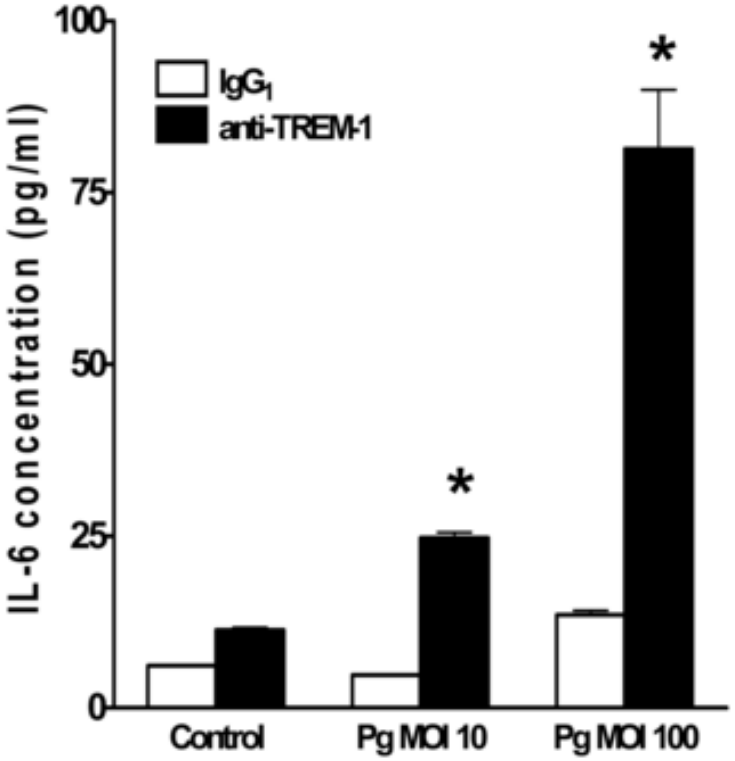


Figure 4


IL-1β concentration (pg/ml)
150
125
100
75
50
25
0
IgG1
anti-TREM-1
*
*
*
Control
Pg MOI 10
Pg MOI 100


IL-6 concentration (pg/ml)
100
75
50
25
0
IgG1
anti-TREM-1
*
*
Control
Pg MOI 10
Pg MOI 100